Calcium-Dependent Neutral Proteinase(Calpain)in Fracture Healing of Rats(Dissertation_全文)

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（ラットの骨折治癒過程におけるカルシウム依存性中性蛋白分解酵素（カルパイン）の働き）

中川泰彰

(2)

Calcium-Dependent Neutral Proteinase (Calpain) in Fracture Healing of Rats

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Summary:

Calpain refers to Ca^{2+}-dependent neutral cysteine proteinase and it was originally thought to be an intracellular proteinase but has recently been shown to be functioning extracellularly as well. This report describes the immunohistochemical demonstration of calpain and biochemical changes in the amount of calpain during the fracture healing events of rats. The tibiae of 6-week-old Wistar rats were fractured, and calluses were obtained from 5 to 28 days after fracture. A frozen section of the fracture callus was stained by the immuno-peroxidase method using polyclonal antibodies of calpains I and II. Positive staining was noted with the anti-calpain II antibody in the perivascular areas, chondrocytes, and cartilage matrix in 5-day, 7-day and 10-day-old calluses. Less intense staining was obtained in older calluses. The caseinolytic activity of calpain II was maximum on the 5th day, high on the 7th and 10th days, and decreased rapidly thereafter. The quantity of calpain II was dependent on the process of fracture healing. It was concluded that calpain was working as one of the matrix proteinases in fracture callus.

Key Words: Calpain-Fracture callus-Matrix proteinase.

Introduction

The biochemical sequence of events during fracture healing is poorly defined, although much is known about the histological aspects of fracture healing. Fracture healing occurs primarily by endochondral ossification in the absence of rigid immobilization and exact apposition of bone ends (17,18,19). During endochondral ossification, proteoglycan content and composition change markedly. In fracture healing, the proteoglycan content is highest at the stage of soft callus and decreases thereafter (18,26), and the size of the proteoglycan aggregate decreases during the early healing

period (14). In the growth plate where bone is formed through endochondral ossification, proteoglycans have been demonstrated to have a definitive change as the calcification in the longitudinal septa of growth cartilage proceeds (5,30). Although there are controversial opinions (24), it is widely believed that proteoglycans have an inhibitory action on calcification by conformational and permiselective effects, excluding negative ion entry and keeping the apparent ion concentration low by keeping the tissue hydrated (3). Therefore, it is supposed that some neutral proteoglycanases exist and promote endochondral ossification.

Calpain, also called calcium-activated neutral proteinase (CANP), is a Ca^{2+}-dependent cysteine proteinase. There are two classes of calpains which differ in their optimal calcium ion concentration for enzymatic activity. Calpain I requires a low concentration of Ca^{2+} for activation, and calpain II requires a much higher Ca^{2+} concentration. Calpastatin is the natural specific inhibitor of calpains I and II. Calpains and calpastatin are known to exist in various cells and tissues, such as, brain, muscle and the submandibular gland (21,23). It is reported that calpains with 80kDa are inactive proenzymes or zymogens and that partially autolysed calpains are active enzymes (11,37). Calpain has been originally thought to be an intracellular proteinase (21). In our previous studies, calpain II was shown to exist in hypertrophic chondrocytes and in the surrounding cartilage matrix of the growth cartilage of rats (31) and to degrade cartilage proteoglycan in vitro (31,34). Calpains I and II were also shown to exist in the synovial fluids of human osteoarthritic knee joints extracellularly (33). It is possible that calpain is working as one of the matrix proteoglycanases, facilitating endochondral ossification in growth cartilage and degrading the cartilaginous matrix in the osteoarthritic joint.

In the present study, we report the immunohistochemical and biochemi-

cal demonstration of calpain II in the fracture callus of rats which is also known to show endochondral ossification.

Materials and Methods

Animal Model of Fracture

Six-week-old male Wistar rats which weighed between 130 and 180 g were used. Under ether anesthesia, the left tibia of each rat was fractured unilaterally by a blunt subcutaneous twisting technique modified from Urist's method (39) using manual pressure against a metal plate. No subsequent fixation or support was applied, and the rats were allowed to move freely in cages. They were then sacrificed, in groups of 3, with deep ether anesthesia after 5, 7, 10, 14, 17 and 21 days. Eighteen rats were used for immunohistochemical studies in total. For biochemical studies, 150 rats were used in total and they were sacrificed after 5, 7, 10, 14 and 28 days, in groups of 30 (Table 1).

Immunohistochemistry

Calpains I and II were purified from porcine red blood cells and the kidneys, respectively, as described previously (8,13). Antisera were raised by repeated injections of rabbits with the respective heavy subunits of calpains I and II with complete Freund's adjuvant. The preparation of these antibodies has been described in detail and these two antibodies have been shown not to be immunologically cross-reactive by immunoblotting (43). The fractured tibia, including the surrounding musculature, was dissected out immediately and quick-frozen in liquid nitrogen. Six μm thick cryostat sections were made and stained by the peroxidase anti-peroxidase (PAP) method (32). Only the 5-day-old specimen was observed with decalcified sections as well as cryostat sections. The detailed method of immunohistochemistry was described elsewhere (31). Control specimens were obtained by incubating with normal rabbit serum or with the anti-calpain II antibody

after preincubation with purified rat kidney calpain II instead of the first antibody. Positive control slides were cut and prepared in the same way from a rat submandibular gland, known to contain calpains I and II, to ensure a working staining system.

Tissue Preparation for Biochemical Study

The fractured tibia was exposed and the fracture calluses were dissected free of muscle and soft tissue. The calluses were carefully scraped off from cortical bone using a blunt chisel. All the following procedures were the same as previously described (31). Briefly, the sample was added to its fourfold volume of 20 mM Tris-HCl buffer, pH 7.5, containing calpain inhibitors, that is, 1 mM glycoletherdiamine tetraacetic acid (EGTA), 1 mM ethylendiamine tetraacetic acid (EDTA) and 5 mM 2-mercaptoethanol. It was homogenized using a Polytron homogenizer (Kriens, Luzern, Switzerland). The callus homogenate was centrifuged at 62,400 g for 90 min in a Hitachi 70P-72 ultracentrifuge using a Hitachi SRP-70AT rotor, and the supernatant was collected. The extract was dialyzed overnight against 20 mM Tris-HCl buffer, pH 7.5, containing calpain inhibitors and 50 mM NaCl, and the aliquot was then applied to a 1.5 by 3.5 cm column of diethylaminoethyl (DEAE)-cellulose (DE 52, Whatman, Springfield, U.K.), preequilibrated with the same buffer. The adsorbed protein was eluted with a linear gradient of 50-500 mM NaCl in a total volume of 100 ml.

Assay of Calpain

Calpain activity was determined as previously described (20), using casein (Hammarstengrade, E. Merck, Darmstadt, Germany) as the substrate. For the assay of calpastatin, a fixed amount of human erythrocyte calpain I or rat kidney calpain II was added and the inhibition of calpain activity on casein was determined as described above. Each fraction of DE 52 chromatography of the callus extract was assayed for calpain and calpasta-

tin.

In order to examine changes in the calpain II activity during fracture healing, 5, 7, 10, 14 and 28 day-old calluses were used. Since soft callus almost disappeared at 28 days, newly formed trabecular bone at the fracture site was collected, and care was taken not to include bone marrow. Right tibial cortical bone which did not include bone marrow was obtained at zero day (control) and muscles around the fracture callus of 5th, 7th and 28th day rats were also collected. The wet weight of calluses, cortical bone and muscle was between 0.615 and 1.77 g. An aliquot of dialyzed extract as calculated to correspond to 0.6 g of wet weight of callus at each time point was applied to DE 52.

Calpain assay was performed in each of the DE 52 chromatographic fractions and fractions which had calpain II activity were collected. Calpain assay was performed again using collected fractions with 5 mM $CaCl_2$ or with the same volume of distilled water. Net calpain II activity was calculated by subtracting the activity in the absence of $CaCl_2$ from that in the presence of 5 mM $CaCl_2$ since calpain activity is dependent on the presence of Ca^{2+}. One unit of calpain was defined as the amount of enzyme that increased the absorbance by 1.0 after incubation at 30° C for 30 min (22). Triplicate analyses were performed at each time point of fracture healing. Statistical evaluations were performed by William's multiple comparison test (41).

Immunoblotting

The dialyzed extract (crude sample) and the fraction of DE 52 chromatography, which had maximum calpain II activity, were applied to immunoblotting. Proteins were subjected to gel-electrophoresis and transferred to a nitrocellulose membrane according to the methods of Towbin et al. (38). Nitrocellulose membranes were cut into strips and some of them were

stained with 0.1% amido black in 40% methanol / 10% acetic acid. The other strips were first incubated with the anti-calpain II antibody and then with a peroxidase-conjugated second antibody directed against the first antibody. Antigens were visualized by the peroxidase staining using 3,3'-diaminobenzidine tetrahydrochloride as substrate (9).

Digestion of Proteoglycan by Calpain II on Histological Sections

Ten-day-old fracture calluses prepared by the same method as the immunohistochemistry were used, because the cartilaginous callus at the 10th day after fracture was the largest of all the stages of the fracture. The serial sections (6 μm thick) were obtained from a fresh frozen block of the callus tissue. Each section was incubated for 1 h at room temperature with 250 μl of enzyme solutions (pH 7.5), and then was stained with safranin-O to demonstrate proteoglycan (28). The enzyme solutions included: 1) 2% hyaluronidase from bovine testes (Sigma, St. Louis, U.S.A.), 2) calpain II from rat kidney 15 U/ml, $CaCl_2$ 5 mM, 3) calpain II 15 U/ml, EDTA 7 mM, 4) Tris-buffered-sodium (TBS) 50 mM (pH 7.2), 5) calpain II 15 U/ml, $CaCl_2$ 5 mM, leupeptin (Peptide Institute, Inc., Osaka, Japan) 8 mg/ml, and 6) calpain II 15 U/ml, $CaCl_2$ 5 mM, leupeptin 80 μg/ml.

Results

Immunohistochemical Demonstration of Calpain

By the present fracture method, the muscles of deep layers were damaged severely and only superficial muscles were intact immediately after fracture. On the 5th day after fracture, the granulation tissues consisting mainly of polymorphonuclear neutrophils, fibroblasts and collagen existed between the cortex of the fractured bones (Fig.1). The findings of 5th day specimens were consistent with those designated as the mesenchymal stage (17).

On the 7th day after fracture, a periosteal callus, a cartilaginous

external callus and fibrous tissues were differentiated on the histological section by a light microscope. In the cartilaginous callus, extracellular matrix proteoglycan existed and some chondrocytes showed hypertrophic changes (Fig. 2). On the 10th day, the bulk of fracture calluses was larger than the 7th day specimen and consisted predominantly of the cartilaginous callus. The cartilaginous callus formed an island mass of hyaline cartilage apart from and between the cortex of the fractured bones (Fig. 3). The findings of 7th and 10th-day specimens were consistent with those designated as soft callus (2). On the 14th and the 17th days, fracture calluses were smaller than those in the stage of soft callus, and the proportion of the cartilaginous callus to cortical bone was smaller. On the 21st day, the size of fracture calluses as well as the proportion of the cartilaginous callus were the smallest of all stages of the present study (Fig. 4). The findings of the 14th, 17th and 21st days were consistent with those designated as hard callus (2).

On the 5th day, positive staining with anti-calpain II was observed in the fibrous and prechondrogenic tissues and the inflammatory cells (Fig. 1). On the 7th day, positive staining with anti-calpain II was primarily extracellular and located almost exclusively in regions of fibrous and prechondrogenic tissues. The positive staining of chondrocyte cells and extracellular matrix around the chondrocytes was sparsely observed (Fig. 2). On the 10th day, deep positive staining with anti-calpain II was noted in the perivascular regions and in the extracellular matrices surrounded by hypertrophic chondrocytes, and there was less intense staining in the chondrocytes as well (Fig. 3). Staining with anti-calpain II on the 14th day and the 17th day decreased significantly as compared to the 7th and 10th day. On the 21st day, positive staining with anti-calpain II was hardly observed, and newly formed trabecular bone showed negative staining with

anti-calpain II (Fig. 4). The positive staining of anti-calpain II was completely blocked when the normal serum was used instead of the first antibody or by a preincubation of the antibody with purified porcine kidney calpain II. Muscle tissue around the fracture callus was positive both with anti-calpain I and anti-calpain II as previously reported (43), and this also served as a positive control for the present study on the fracture callus.

In the high power magnification of the 10-day fracture callus, the cytoplasm of chondrocytes in cartilaginous lacunae showed positive staining with anti-calpain II when the counterstaining with methyl green was deep (Fig. 5A). In those sections which were very lightly counterstained with methyl green, the extracellular matrices adjacent to the hypertrophic chondrocytes as well as cytoplasmic areas of the hypertrophic chondrocytes showed positive staining with anti-calpain II. However, these stainings were patchy and did not stain around all hypertrophic chondrocytes (Fig. 5B). The perivascular areas stained with anti-calpain II were also observed (Fig. 5C). In the hypertrophic chondrocyte region, the perivascular staining was predominant. Calpain I was not demonstrated in all the stages of the fracture in the present study.

Biochemical Demonstration of Calpain

Figure 6 shows a DEAE-cellulose chromatogram obtained with the crude cartilaginous extract of 14-day fracture callus. The peak of calpastatin or calpain inhibitory activity was obtained at the 150 mM NaCl gradient as a decrease of casein-degrading activity, when a fixed amount of purified calpain II from rat kidney was added to each assay tube, and the activity of calpain was determined in the presence of 5 mM Ca^{2+}. Just after the calpastatin fractions, a positive peak of activity, calpain II activity, was noted at the 330 mM NaCl gradient. In all the stages of fracture callus of which calpain II activity was detected, the similar chromatographic pattern

of DEAE-cellulose was obtained.

Immunoelectrophoretic Blotting of Calpain from Fracture Callus

The crude sample of 7-day fracture callus as well as the combined elution fractions from the DEAE-cellulose column of the same stage which had maximum calpain II activity were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. When the immunoblotting was carried out with the anti-calpain II antibody, a single band of Mr 80 kDa (Fig. 7, arrowhead) appeared with the crude extract (Fig. 7, lane C) and DEAE fractions (Fig. 7, lane D). In all the stages of fracture callus which contained calpain II activity, a single band of Mr 80 kDa against anti-calpain II antibody was demonstrated.

Changes in the Calpain II Activity during Fracture Healing

The calpain II activity of the fracture callus reached the maximum at the 5th day after fracture. The calpain II activities of the 7-day and 10-day fracture calluses were nearly the same level and were slightly lower than that of the 5th day. The calpain II activity of the fracture callus decreased rapidly thereafter. The calpain II activity was not detected in the 0-day and 28-day fracture calluses. The activity of calpain II in the muscles around the calluses was lower than that of the fracture callus at every time point of fracture healing and higher than the new bone tissue after fracture healing (28 day) (Table 2).

Digestion of Proteoglycan by Calpain II on Histological Sections

Intense staining with safranin-O was noted after incubation with TBS (enzyme blank) (Fig. 8A). However, after calpain II incubation with Ca^{2+}, staining with safranin-O decreased significantly (Fig. 8B). The similar decrease of staining was obtained when a hyaluronidase solution was applied on the histological section. When calpain II was incubated in the absence of Ca^{2+}, the staining with safranin-O was obtained as the control section.

After incubation with calpain II, Ca^{2+} and excessive amounts of leupeptin, cysteine protease inhibitor, the staining with safranin-O remained as intense as the control (enzyme blank). When calpain II was incubated with Ca^{2+} and with insufficient amounts of leupeptin, safranin-O staining was less intense than the control (Table 3).

Discussion

Nonrigidly fixed fractures heal by a process of endochondral ossification (17,18,19). Lane et al. differentiated four stages of endochondral fracture healing: 1) mesenchymal, 2) chondroid, 3) chondro-osteoid, and 4) osteogenic (17). The alteration of several enzymes in fracture repair is reported as follows. Alkaline phosphatase peaked during the chondro-osteoid stage and declined before and after this stage (12). Its enzyme is used as the marker enzyme for the callus calcification (40). Aminopeptidase and β-naphthylamidase increased during the mesenchymal stage and then decreased. These enzymes are involved in the process of vascular invasion in the vicinity of the fracture (25). Enzymes mediating carbohydrate metabolism remained relatively constant throughout the entire fracture healing process (15).

Since the first identification of a Ca^{2+}-dependent cysteine proteinase (calpain) from rat brain (7), calpain activity has been demonstrated in many cells and tissues from a variety of species (21,23) and it is known to be one of the most significant intracellular nonlysosomal proteases. Two forms of calpain are known to exist which differ in the Ca^{2+} requirement for optimal activity. Calpain I is activated by μM concentrations of Ca^{2+} whereas calpain II requires mM amounts of Ca^{2+} for activity. Both enzymes are heterodimers, each being composed of one heavy (approximately 80 kDa) and one light (approx. 30 kDa) subunit. Calpain-specific endogenous inhibitor protein, called calpastatin, is as widely distributed among mammalian

and avian cells as calpain.

In the present study, we demonstrated immunohistochemically that the fibrous tissues, the perivascular regions, hypertrophic chondrocytes and the extracellular matrices surrounded by hypertrophic chondrocytes showed positive staining with anti-calpain II in the fracture callus of Wistar rats. The peak fractions having enzymatic activities of DEAE-cellulose chromatography were found to contain calpain II protein as distinctly demonstrated by immunoelectrophoretic blotting with anti-calpain II antibody. The positively stained single band appeared at a position for 80 kDa on SDS-gel, which is identical with the size of the heavy subunit of calpain from growth plate (31) and other tissues of rats (42). The result of immunoelectrophoretic blotting, in turn, verified the specificity of the immunohistochemical studies since no other proteins in the cartilaginous extract showed a positive reaction with the same antiserum which was used in immunohistochemistry. Caseinolytic activity obtained from fracture callus was demonstrated to be derived from calpain II by its elution position on an ion-exchange chromatography, immunoelectrophoretic blotting with anti-calpain II antibody and calcium dependency. When rat kidney calpain II was activated on the fresh sections of the calluses which contained proteoglycan, stainability with safranin-O decreased significantly.

Calpains have generally been known as intracellular proteinases. However, in our recent studies, calpain II was demonstrated to be present in the cartilaginous extracellular matrix and chondrocytes in the hypertrophic zone of the growth cartilage of new born Wistar rats (31). Calpain I and II and calpastatin were shown to exist in the synovial fluids of human osteoarthritic knee joints (33) and rheumatoid arthritic knee joints (6). Calpains degraded cartilage proteoglycan in vitro (31,34). Kumamoto et al. recently demonstrated an extracellular localization of calpain in the

skeletal muscle after sciatic denervation or starvation of rats by immunogold electronmicroscopy (16). It was also reported that calpain II was distributed in the extracellular matrix of skeletal muscle, lung and aorta under physiological conditions (1). This data suggests that calpain may function in extracellular space in certain conditions. In the present study, calpain II was shown to be present in the perivascular regions and the chondrocytes and the extracellular matrices surrounded by hypertrophic chondrocytes. Therefore, our present data supports the concept that calpain II could work as matrix proteinase.

Our present study showed calpain II activity was highest at the 5th day designated as the mesenchymal stage. Immunohistochemically most of calpain II at this stage was contained in the extracellular fibrous tissues and the inflammatory cells. The function of calpain II at this stage of fracture healing is unknown, although some reactive emergence of calpain against inflammation is implicated (29). On the 10th day, positive staining with anti-calpain II was also observed in the perivascular regions. It was reported that calpain was identified in vascular endothelial cells (27) and small vessels (10). Though it was suggested that calpain in the vascular endothelial cells activated coagulation (27), the function of calpain II in the perivascular regions of fracture healing is not elucidated.

The results of calpain digestion on histological sections as shown in Figures 8A and B and Table 3 showed that calpain II degraded cartilage proteoglycan under artificial conditions. Because the same results of histological digestion were obtained when the calpain was applied after preincubation at 60° C of histological sections, it was unlikely that the loss of staining with safranin-O resulted through an activation of latent proteases (data not shown). This result was coincident with our report that calpains degrade cartilage proteoglycan in vitro (31,34). Einhorn et al.

reported neutral protein-degrading enzymes in experimental fracture callus (4). Their endopeptidases were measured in the presence of 1.5 mM $CaCl_2$ and in the absence of calpain inhibitors. Their results do not seem to include calpain activity since calpain is vulnerable to autolysis and decreases its activity rapidly in the presence of Ca^{2+} (35,36). Our present study does neither support nor deny the possible function of the reported proteoglycanase, but our results demonstrated that calpain II may be one of the proteoglycanases in the chondroid and chondro-osteoid stages of fracture repair.

Calpain I was not demonstrated immunohistochemically in fracture callus. The anti-calpain I antibody used in the present study gave positive immunostainings of rat submandibular glands as had been reported (43). Although calpastatin was detected in every chromatogram of fracture callus between 5 and 14 days after fracture, the amount of this inhibitor protein was inconsistent with the fracture healing process. The significance of calpastatin in fracture callus is yet to be investigated.

Finally, immunohistochemically and biochemically the quantity of calpain II was dependent on the process of fracture healing, that is, it increased at the stage of mesenchymal and soft callus and decreased at the stage of hard callus and remodeling. Our results also clearly demonstrated that calpain II was functioning as one of matrix proteinases in fracture healing, although the precise nature of its substrate was not elucidated.

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Figure Legend

Figure 1:

Immunohistochemical demonstration of calpain. Calpain was stained by the immunoperoxidase method, detected by the development of peroxidase staining using 3,3'-diaminobenzidine tetrahydrochloride as the substrate, and counterstained by methyl green. Fracture healing on Day 5. A decalcified section. Positive staining with anti-calpain II was obtained in the fibrous tissues (arrow) between the cortex of the fracture bones (arrowheads). (x 48)

Figure 2:

Fracture healing on Day 7. The following histological figures were frozen sections. Positive staining with anti-calpain II was observed in the region of fibrous tissues (arrow). Positive staining was also obtained in the chondrocytes (arrowheads) of the cartilaginous callus near the cortex of the fracture bone. (x 120)

Figure 3:

Fracture healing on Day 10. Positive staining with anti-calpain II was noted in the perivascular regions (large arrow) and in the extracellular matrices (small arrows) surrounded by hypertrophic chondrocytes and in the chondrocyte (arrowheads) as well. (x 120)

Figure 4:

Fracture healing on Day 21. Staining with anti-calpain II was hardly observed in the interstitium and newly formed trabecular bone at the right of the figure showed negative staining. (x 120)

Figure 5:

Photomicrographs with high-power magnification of the cartilaginous callus on Day 10. **A:** When the counterstaining with methyl green was deep, the cytoplasm of hypertrophic chondrocytes showed positive staining with

anti-calpain II (arrows). (x 430) **B:** When the counterstaining with methyl green was made light, the extracellular matrices (arrows) adjacent to the hypertrophic chondrocytes as well as areas of the hypertrophic chondrocytes (arrowheads) showed positive staining with anti-calpain II. But, it did not stain around all hypertrophic chondrocytes. (x 560) **C:** The perivascular areas stained with anti-calpain II were also observed. (x 560)

Figure 6:

DEAE-cellulose chromatogram of the crude cartilaginous sample of 14-day fracture callus. Crude extract from rat fracture callus (wet weight 0.87 g) was dialysed and applied to the column (1.5 by 3.5 cm) of DEAE-cellulose (DE 52), which was eluted with a linear gradient of 50-500 mM NaCl in a total volume of 100 ml. Fractions of 2.0 ml for each tube were collected. Calpain activity was measured as caseinolytic activity, and calpastatin activity was measured as caseinolysis when a fixed amount of rat kidney calpain II (0.35 U) was added to each tube. Horizontal arrows indicate fractions used for the study of the calpain II activity during fracture healing (see Table 2).

Figure 7:

Immunoelectrophoretic blotting of calpain from 7-day fracture callus by anti-calpain antibodies. A portion of the crude cartilaginous extract (lanes A, C) and the calpain fractions from DEAE-cellulose column shown in Fig. 6 (lanes B, D) and protein standards were subjected to 10% SDS-polyacrylamide gel electrophoresis, and then transferred to a nitrocellulose membrane which was stained with amido black (lanes A, B) or immunoblotted with anti-calpain II antibody (lanes C, D). The migration positions of the marker proteins used are shown on the left (using phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase and soybean trypsin inhibitor as molecular markers): kDa, kilodalton. Arrowhead indicates the position

calculated for 80 kDa.

Figure 8:

Digestion of proteoglycan by calpain II on histological sections. The serial sections of 10-day-old fracture calluses were used. Each section was incubated for 1 h at room temperature with the same volumes of enzyme solutions (pH 7.5), and then was stained with safranin-O. **A:** Intense staining with safranin-O was noted after incubation with TBS (enzyme blank) (x 120). **B:** After incubation with calpain II in the presence of Ca^{2+}, the staining of safranin-O decreased significantly (x 120).

TABLE 1

Experimental design

Days after fracture	(0)	5	7	10	14	17	21	(28)
Immunohistochemical study*	-	3	3	3	3	3	3	-
Biochemical study*	(30)	30	30	30	30	-	-	(30)

* number of animals used for the present study

( ) For reference, cortical bone of the unfractured site was collected at day zero and newly formed trabecular bone at the fracture site was collected at the 28th day.

TABLE 2

Calpain II activity of the external callus during fracture healing in the tibia of rats (Mean and standard deviation)

Healing time (days)	Calpain II activity* in fracture callus (units / g)	Calpain II activity in muscle** (units / g)
0	ND***	
5	3.84±0.54	1.40
7	3.18±0.47	1.84
10	2.76±0.20	
14	1.85±0.62	
28	ND***	0.87

* Calpain II activity was measured in units/g wet weight of tissue. n=3. All fractions between the two arrowheads in Fig. 6 were collected and measured with caseinolytic activity.

** Muscles in contact with the fracture callus were collected and calpain II activity was measured.

*** ND = not detected

! $0.01<P\leq0.05$

!! $P\leq0.01$

TABLE 3

Degradation of proteoglycan with calpain II

on histological sections of the fracture callus

Enzyme solutions	Safranin-O staining
Hyaluronidase	-
Calpain II + Ca^{2+}	-
Calpain II + EDTA	++
TBS (control)	++
Calpain II + Ca^{2+} + Leupeptin (8mg/ml)	++
Calpain II + Ca^{2+} + Leupeptin (80μg/ml)	+

- : no staining with safranin-O

+ : positive but less intense staining with safranin-O

++ : intense staining with safranin-O

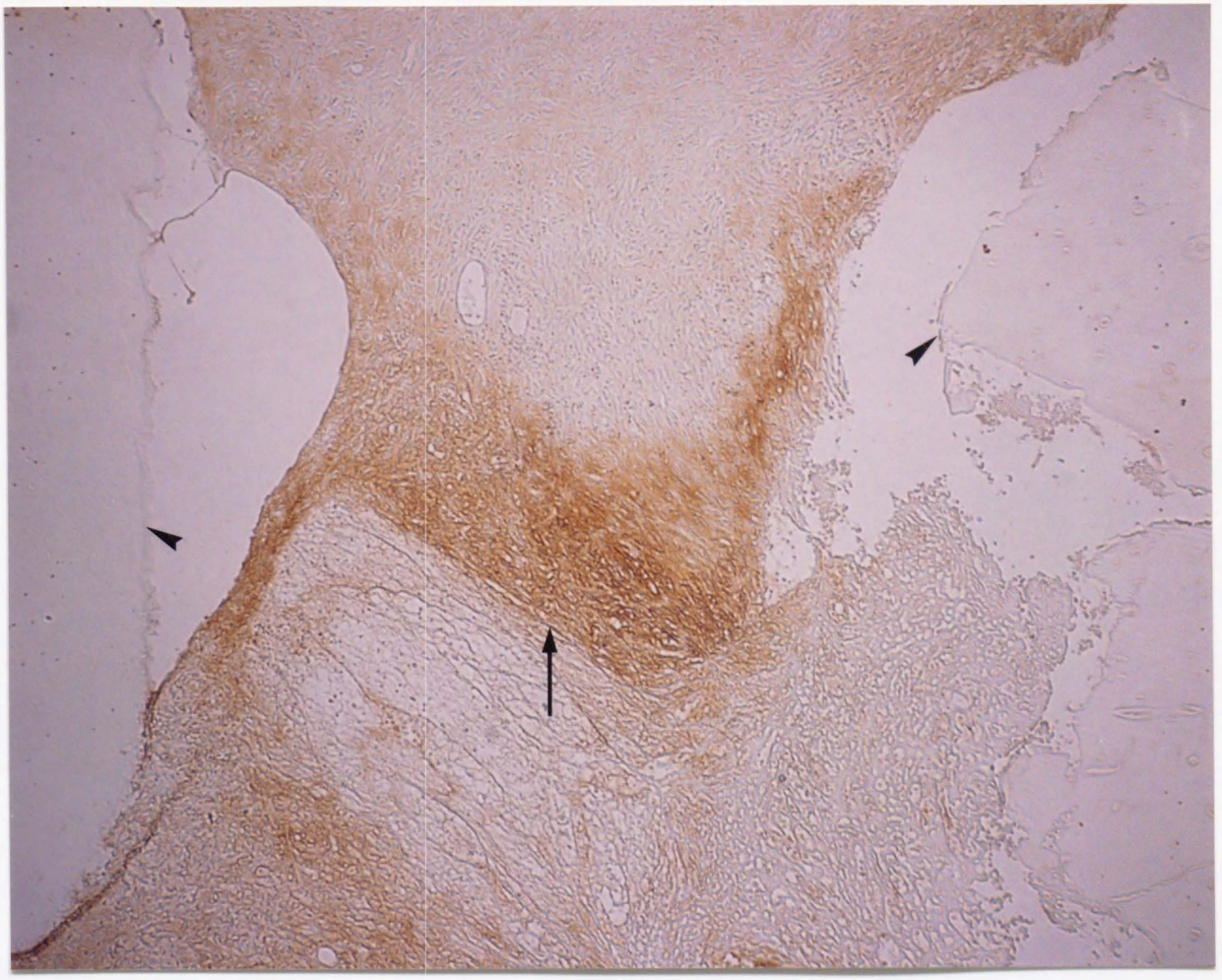

Fig.1

Fig.2

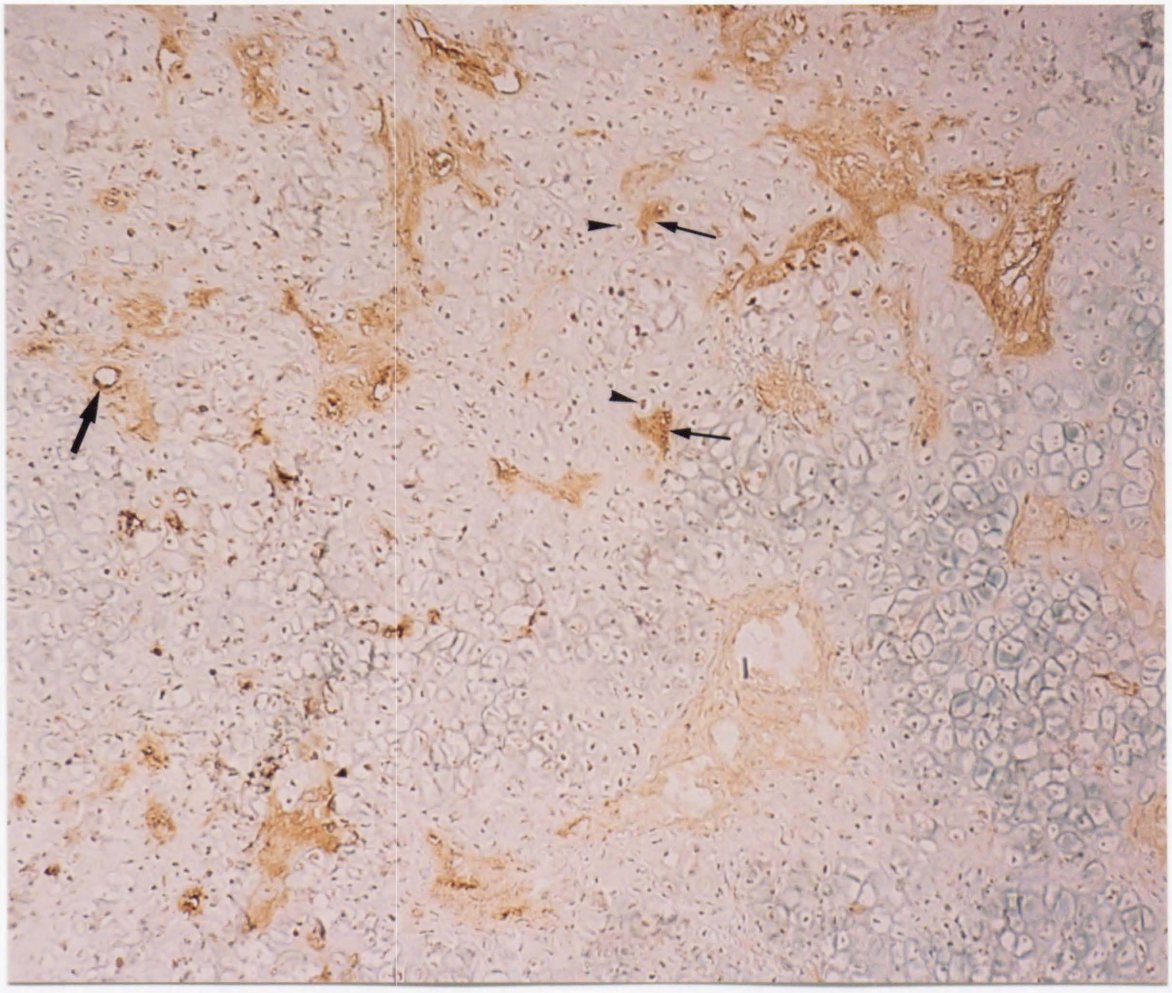

Fig.3

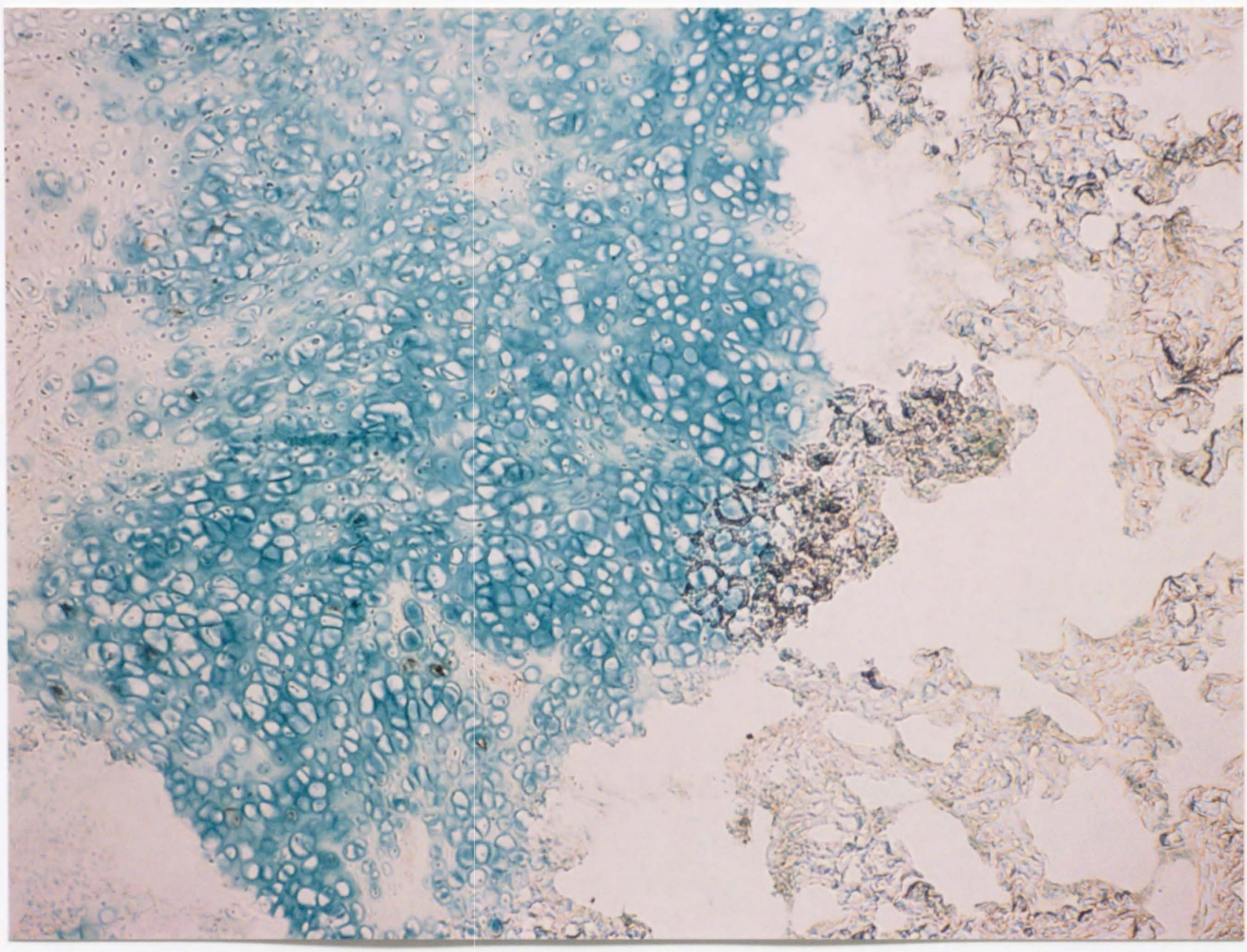

Fig.4

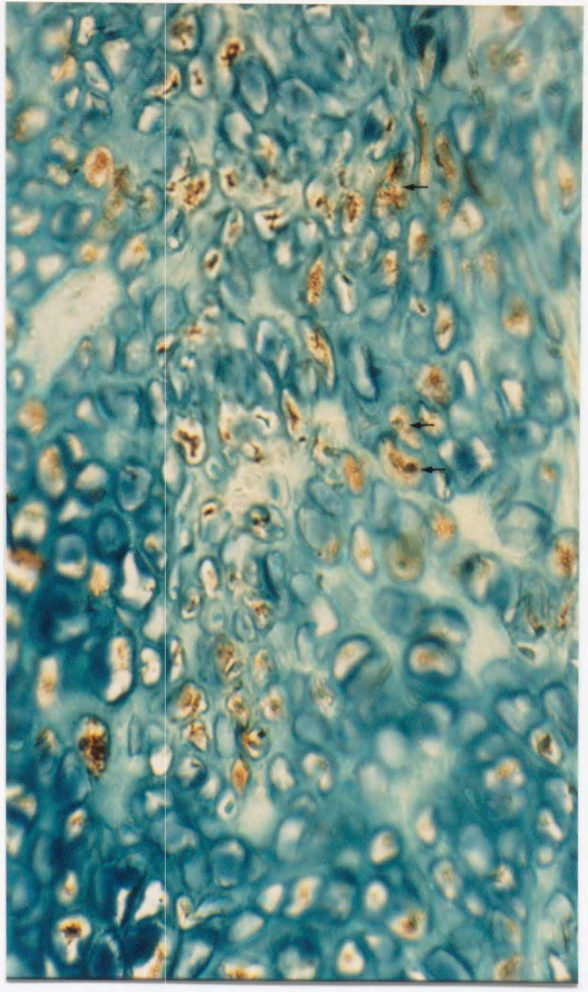

Fig.5A

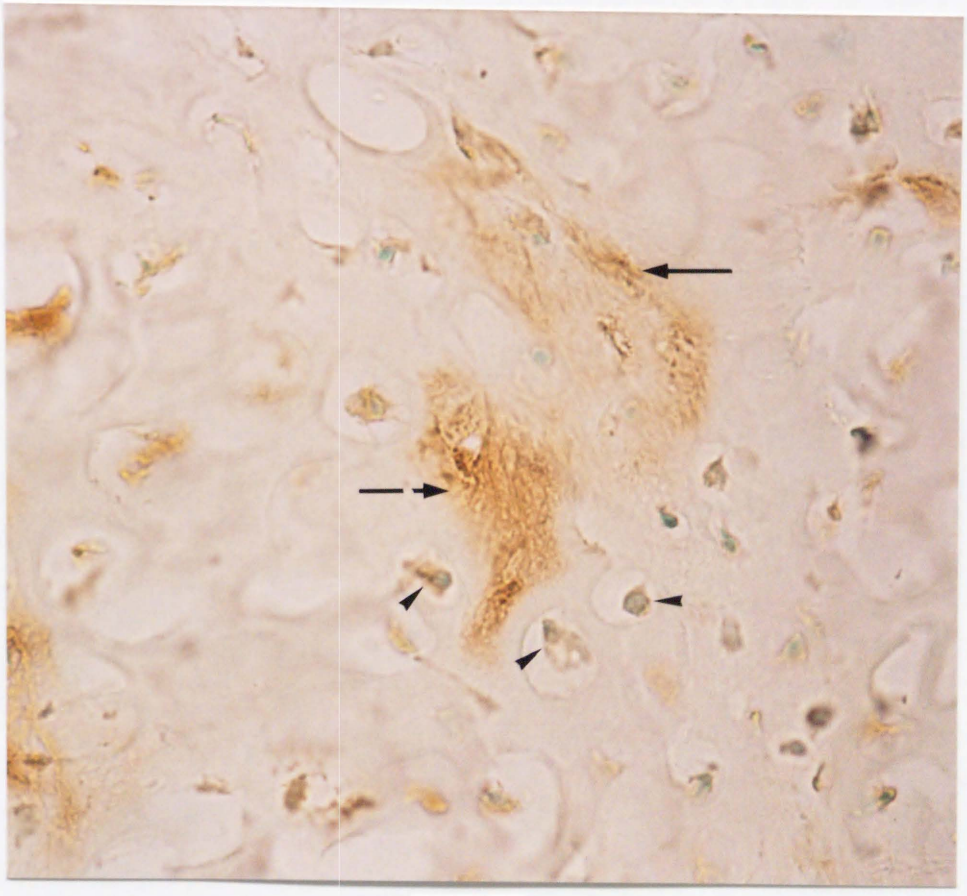

Fig.5B

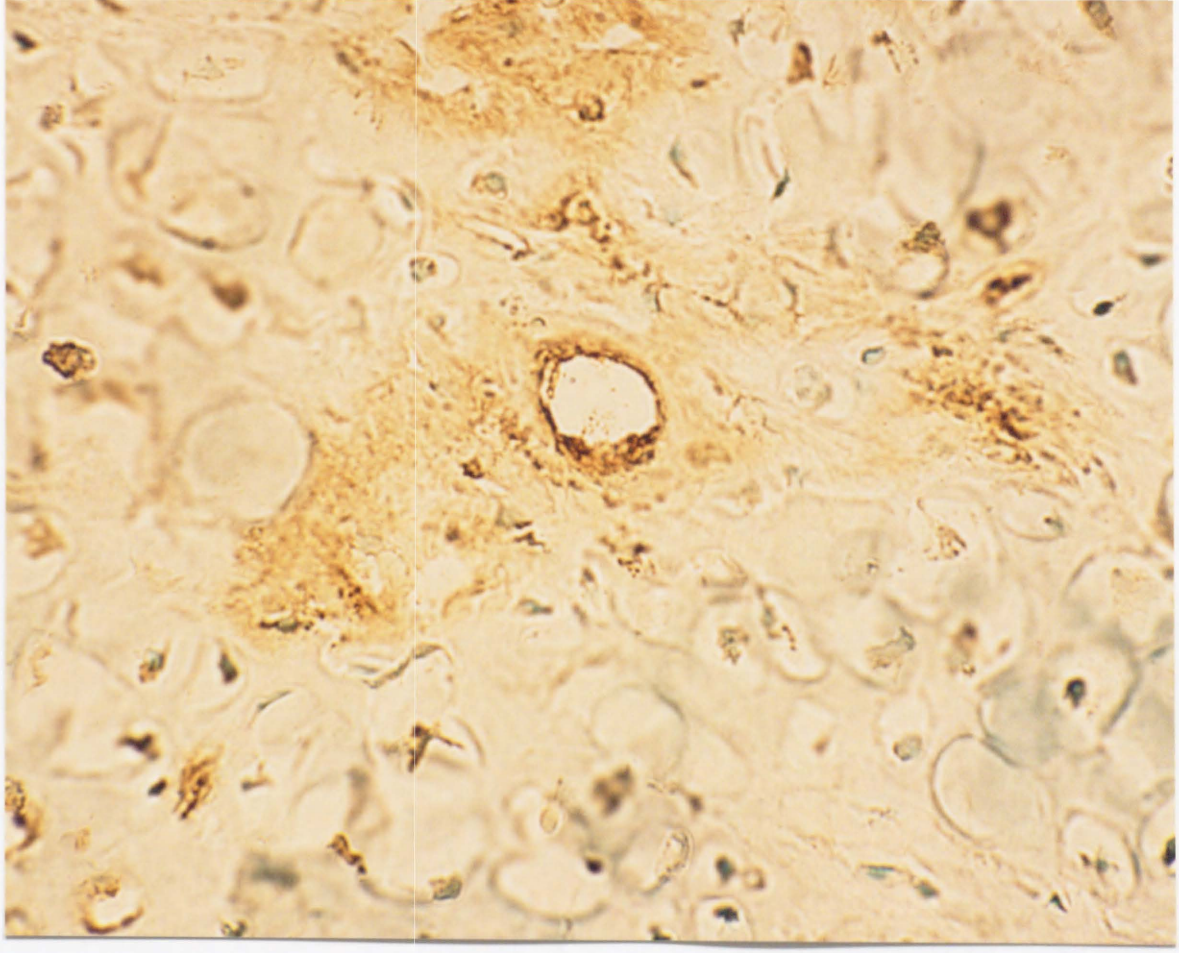

Fig.5C

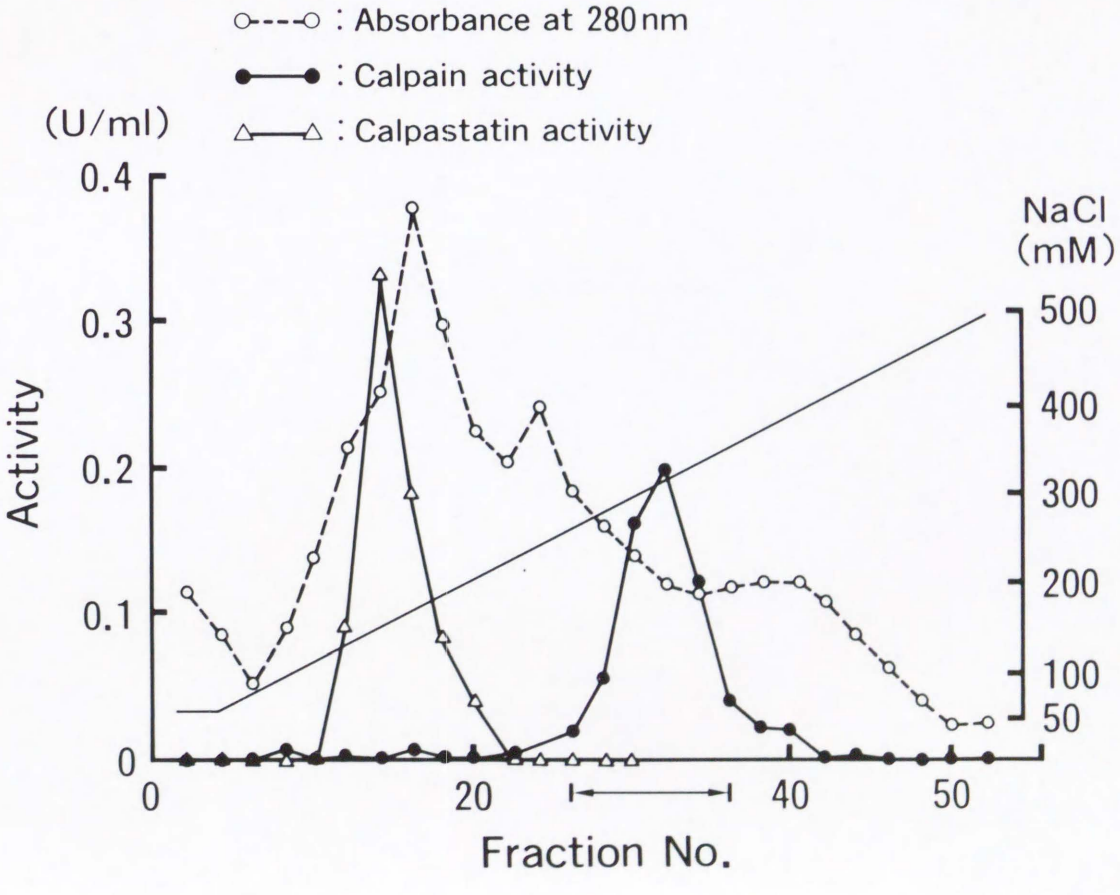
○---○ : Absorbance at 280nm
●——● : Calpain activity
△——△ : Calpastatin activity
(U/ml)
0.4
0.3
0.2
0.1
0
Activity
NaCl
(mM)
500
400
300
200
100
50
0
20
40
50
Fraction No.

Fig.6

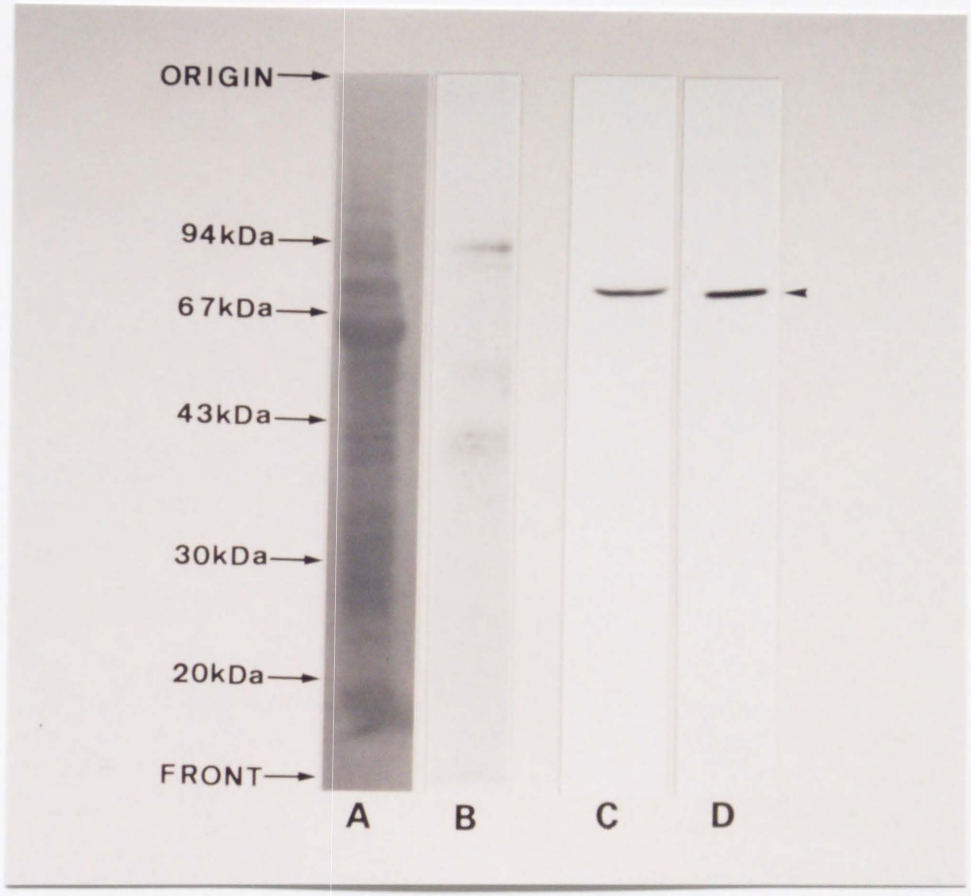

Fig.7

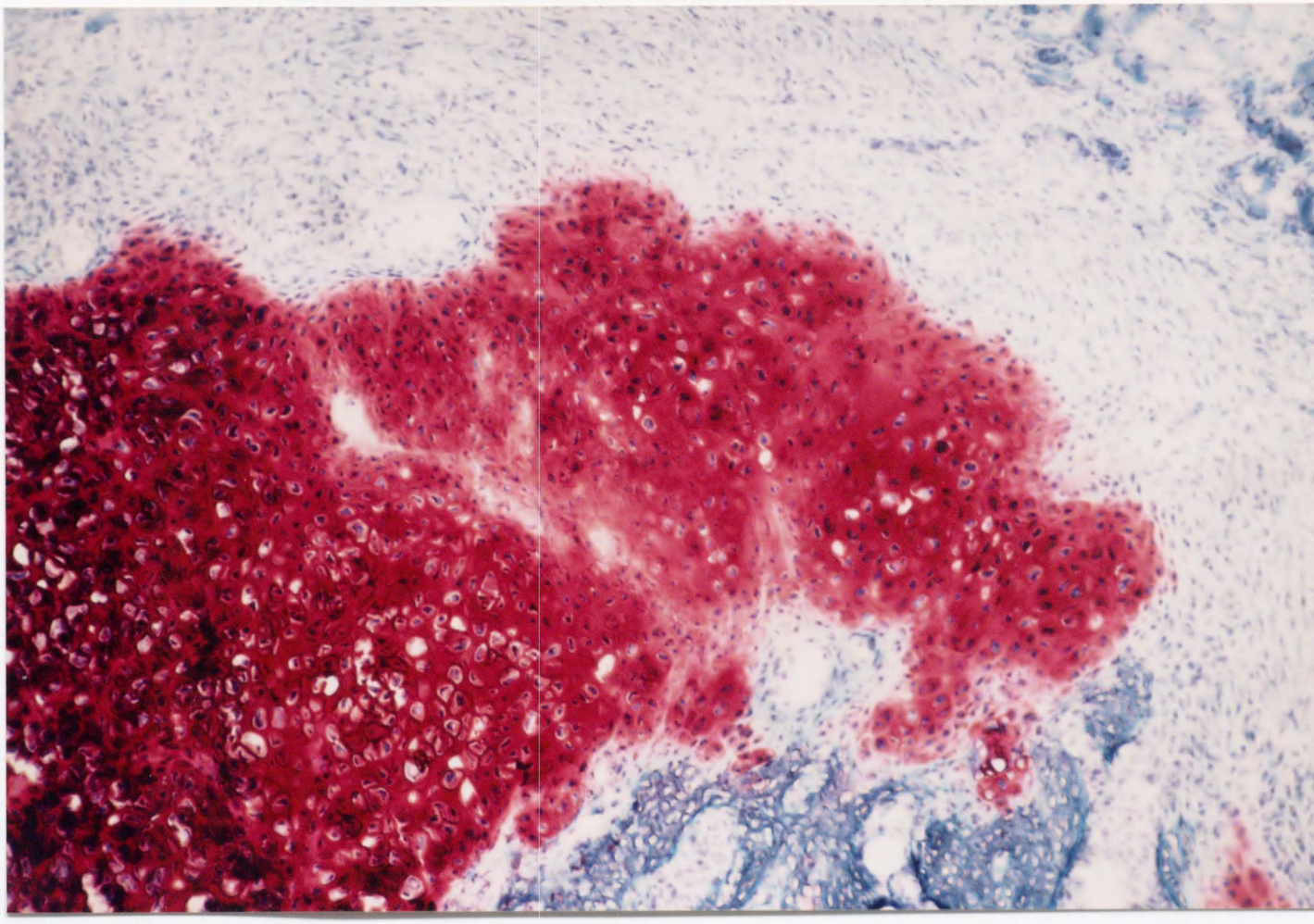

Fig.8A

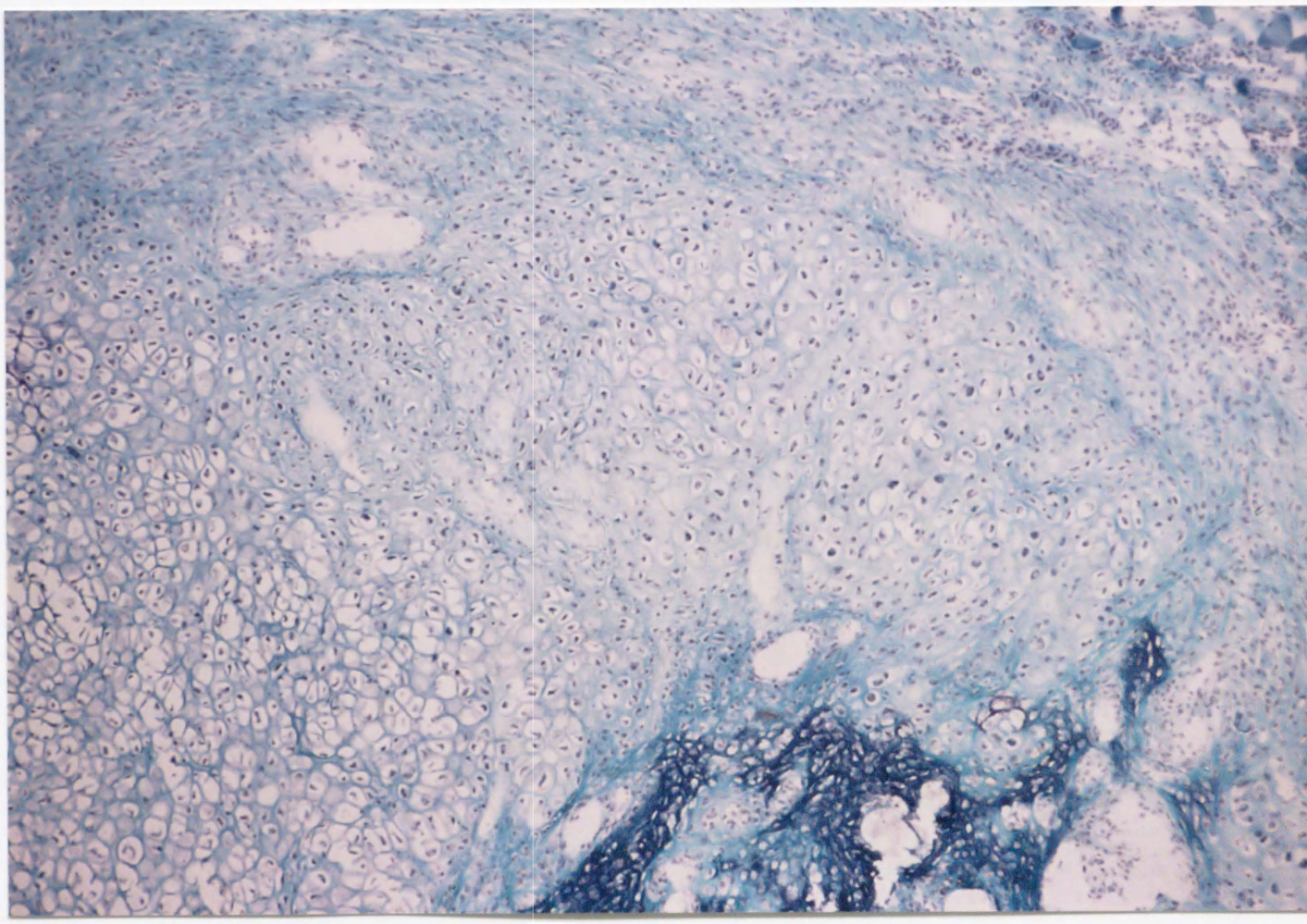

Fig.8B